Avian hind-limb digit length ratios measured from radiographs are sexually dimorphic

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Abstract

Sexual dimorphism in digit length ratios is well established in humans, and has been reported in other vertebrate species as well, including birds. The sign of sexual dimorphism in digit ratios may, however, vary both within and between vertebrate classes. It has been hypothesized that sex differences in digit ratios arise via differential prenatal exposure of the two sexes to steroids, which may affect the expression of the *Hox* genes controlling the osteometric development of digits and appendices. Among birds, the evidence for sex dimorphism in hind-limb digit ratios is conflicting, though all previous studies were based on measurements of undissected digits, implying that results could be confounded by sex-related variation in soft tissues. Here we report that digit ratios derived from radiographs of both feet of a large passerine bird, the hooded crow (*Corvus corone*), are sexually dimorphic, males showing larger 2D : 3D (effect size, $r = 0.33$) and 2D : 4D than females (effect size, $r = 0.28$). We also observed a good agreement ($r = 0.45$) between radiographic estimates of digit ratios and digit ratios calculated based on undissected digit measurements (thus including soft tissues). Importantly, we found that the patterns of sex and side differences were largely coherent between the two methods. Therefore, our findings show for the first time in avian species that sex differences in digit ratios have an osteometric basis, a fundamental prerequisite for a role of *Hox* genes in originating such dimorphism.

Key words 2D : 4D; *Corvus corone*; digit ratio; passerines.

Introduction

Subtle sex differences in the relative lengths of fingers have long been reported in humans, men usually bearing a relatively longer ring (4D) compared to index (2D) finger than women (reviewed in Peters et al. 2002). This results in men having smaller 2D : 4D ratios than women, a pattern which appears to be consistent across different ethnic groups, despite ethnic variation in digit ratio values (Manning, 2002; Manning et al. 2004, 2007). Sex differences in digit ratios extend to other digit ratios and to ratios between metacarpal bones (McFadden & Shubel, 2002; Manning et al. 2003; Saino et al. 2006a; Robertson et al. 2008), and directional asymmetry in 2D : 4D has been reported in some studies, the right side showing lower ratios than the left (Manning et al. 1998; Williams et al. 2000; reviewed in Manning, 2002). Individual 2D : 4D ratio in humans is established as early as during the 14th week

of gestation (Garn et al. 1975) and remains consistent within individuals, as shown by longitudinal studies (McIntyre et al. 2005; Trivers et al. 2006), though it may show minor variation in women through the menstrual cycle (Mayhew et al. 2007).

Intersexual as well as interindividual differences in digit ratios could arise as a consequence of variation in the hormonal environment *in utero* in terms of sex steroids, as originally suggested by Manning et al. (1998). This could occur because the development of fingers and appendices is controlled by *Hox* genes, which also influence the development of the urogenital system (Manning et al. 1998; McIntyre, 2006). For example, the hand-foot-genital syndrome, which is caused by a mutation of *Hox* genes, results in morphological as well as functional anomalies in fingers, toes and the urogenital system (Mortlock & Innis, 1997). Therefore, Manning et al. (1998) proposed that sex differences in embryonic hormone production or exposure to maternal hormones could affect the development of genitalia as well as the length of appendices differently in the two sexes, which could explain sexual dimorphism in digit ratios. Accordingly, variation in the intrauterine hormonal environment, as assessed by the relative concentration of testosterone (T) to estradiol (E) in amniotic

samples, correlates negatively with the 2D : 4D ratio of children of both sexes at 2 years of age, implying that a higher exposure to prenatal androgen relative to estrogen levels is associated with a more 'masculine' digit ratio (Lutchmaya et al. 2004). Moreover, as hormones have important activational and organizational effects on morphological, psychological and performance traits (including pathological conditions), a large body of literature suggested that digit ratios could serve as phenotypic markers of prenatal exposure to androgens and estrogens, by predicting variation in such traits (for recent studies, see e.g. Manning, 2002; Bailey & Hurd, 2005; Fink et al. 2005; Kempel et al. 2005; Paul et al. 2006; Romano et al. 2006; Saino et al. 2006b; Zhang et al. 2008; but see Putz et al. 2004).

Sex differences in relative digit lengths have also been reported in non-human primates, mice, birds and reptiles (e.g. Brown et al. 2002; Burley & Foster, 2004; Roney et al. 2004; Leoni et al. 2005; Rubolini et al. 2006; Saino et al. 2007). This could occur because *Hox* genes are highly conserved across vertebrate classes (Manning, 2002). However, the evidence for sex differences in digit ratios among birds, as evaluated by external measures of undissected digits, is conflicting (e.g. Burley & Foster, 2004; Forstmeier, 2005), and may be confounded by sexual dimorphism in the extent of soft tissues on the digits. Perhaps surprisingly, to date no study has addressed whether sexual differences actually exist in skeletal digit length ratios by measuring the bones either from dissected digits or from radiographs. This is important because *Hox* genes are known to influence skeletal growth rather than soft tissue development (Tickle, 2004), and therefore the occurrence of skeletal sex differences is a prerequisite for a role of *Hox* genes expression in affecting sexual dimorphism in digit ratios (Manning, 2002).

The aim of this study was therefore to fill this gap by analysing sex- and side-related variation in hind-limb digit ratios (2D : 3D, 2D : 4D and 3D : 4D) in a large passerine bird, the hooded crow (*Corvus corone*), based on radiographs of the feet. The methods adopted to quantify avian hind-limb digit ratios in previous studies have included a variety of techniques involving direct measurements of the digits or indirect measurements of, for example, footprints (see Burley & Foster, 2004; Forstmeier, 2005; Romano et al. 2005; Navarro et al. 2007), but there are no studies reporting either direct or indirect bone measurements. Similarly, few studies conducted in non-human species cross-validated different methods to obtain digit ratio estimates (e.g. Forstmeier, 2005), and none has assessed the correlation of digit ratios obtained from whole digits and bone measurements. Therefore, we compared digit ratio estimates as obtained from standardized external measurements of whole fingers (thus including ligaments and soft tissues) with those derived from radiographic bone measurements.

Methods

Specimens

Digit measurements were gathered from corpses of 70 adult hooded crows that were culled by local administration authorities during routine crow population control activities in the Po plain (NW Italy) in 2006–2007. Whole legs were separated from the body and kept frozen until measurement. Corpses were sexed both anatomically (by gonadal inspection) and molecularly following established procedures (Griffiths et al. 1998), as a part of another project (F. Haas and N. Saino, unpubl. data).
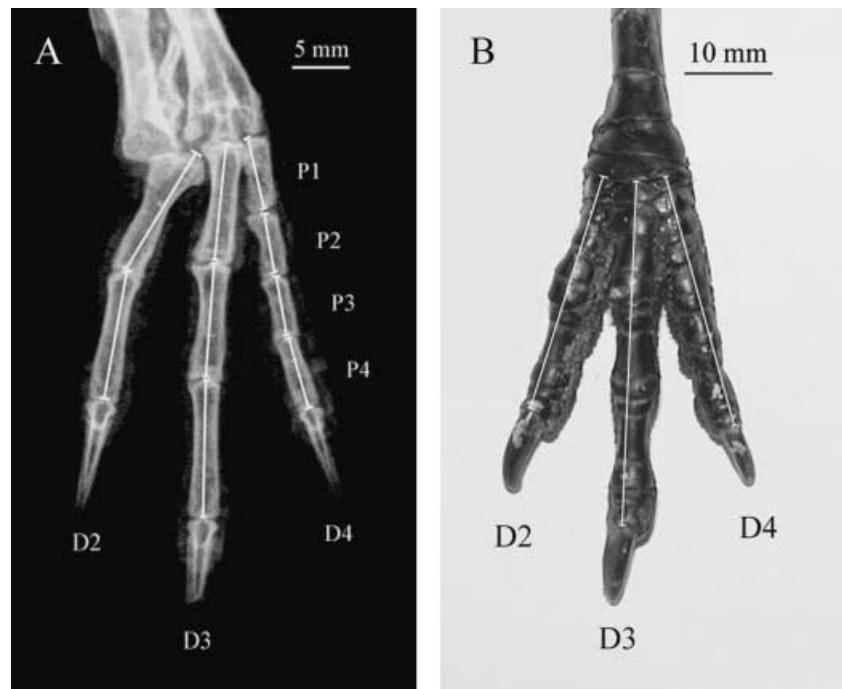
Radiographic measurements

Phalanges were measured from radiographs of the foot. Six pairs of legs (right and left) were fixed with tape in a standard position (plantar side upwards) onto a 30×20-cm cassette containing a photostimulatable phosphorus plate, which was exposed to a stimulating X-ray beam (16 mAs and 42 kV) from a distance of 100 cm. A 30-kW radiation apparatus (Arcom s.r.l., Vimercate, Milan, Italy) was used. Digital radiographic images (DICOM format) were recorded using a computer system (Agfa COMPACT) and were analysed by means of the EFILM™ software (ver. 2.1.2; Merge Technologies Inc., 2006). A reference ruler was included in all tables. Measures of individual phalanges were recorded (in pixels) based on standard proximal and distal reference landmarks (Fig. 1). Right foot images were rotated on their vertical axis prior to measurement to avoid side-related biases in measurements. The length of the whole digit was obtained by summing up the lengths of individual phalanges for each digit.

The repeatability of radiographic estimates of digit ratios (hereafter identified by RAD), assessed separately for each digit ratio and side, was evaluated based on pairs of legs that were radiographed twice, taken on 13 randomly selected individuals. The repeatability (R) was very high, being > 0.68 for 2D : 3D ($F_{12,13} > 5.33$, $P < 0.003$) and > 0.94 for the other digits and sides ($F_{12,13} > 31.7$, $P < 0.0001$).

Measurements of whole digits

Whole digits were also measured directly by means of digital callipers (accuracy 0.01 mm). Measurements were taken while hanging the leg with the fingertips downwards. A standard weight (300 g) was applied by means of a clip to the nail of each digit prior to taking the measurement, to properly stretch the digit. Measurements were taken dorsally from a proximal landmark, identified as the distal margin of the first undivided dorsal scale covering the tarsal bones, which lies approximately over the joint between the proximal phalanx and the tarsus, to a distal landmark, identified as the proximal end of the nail (excluding toenail) (Fig. 1). Whole measurements of each digit were taken twice and the mean value was used in subsequent analyses.

Similarly to RAD digit ratios, the repeatability of digit ratios obtained from whole digit measurements (hereafter WHO), as assessed on pairs of legs of a sample of 17 randomly selected individuals that were measured two times by repeating the whole preparation procedure each time, was also very high, being > 0.94 in all cases ($F_{16,17} > 35.9$, $P < 0.0001$).

Fig. 1 (A) Landmarks used for radiographic measurements of phalanx lengths. The numbering of individual phalanges (P1 to P4) is shown, as well as the digit number (D2 to D4). (B) Landmarks used for measurements of whole digit lengths. The numbering of individual digits (D2 to D4) is shown.

Statistical analyses

The correlation between RAD and WHO digit ratios was assessed by means of the Pearson's correlation coefficient (r) calculated for each digit ratio × side × sex combination ($n = 12$ correlation coefficients). In addition, we tested whether the correlation between WHO and RAD digit ratios differed between the sexes and sides by means of an analysis of variance, where the 12 correlation coefficients (Zr-transformed; Rosenthal, 1991) were the dependent variable, and sex, side and their interaction were the predictors.

Sex- and side-related differences in digit ratios were analysed by means of mixed models, separately for each digit ratio, with individual as a random factor, and side (right and left), sex and their interaction as fixed effects. The interaction term between side and sex was removed as its effect was always non-significant (RAD models, all $P > 0.24$; WHO models, all $P > 0.17$). Given that there is no standard way to calculate effect size for mixed models (Nakagawa & Cuthill, 2007), we expressed it as the standardized regression coefficient of a model where the digit ratio of interest was standardized to a mean of 0 and a standard deviation of ±1, and where both sex and side were included as standardized covariates. Means and estimates are presented together with their associated 95% confidence limits (CL). We did not apply any experiment-wise correction to P-values because such corrections may not be suitable for digit ratio studies (see Leoni et al. 2005 for a thorough discussion; see also Nakagawa, 2004 for a critique of the use of Bonferroni corrections).

RAD digit ratios could be obtained from 68 individuals (35 males, 33 females), and WHO measurements could be taken on 65 individuals (30 males, 35 females). Minor discrepancies in sample size are due to damaged fingers or bones, which impaired accurate measurements of individual digits.

Results

Correlations between RAD and WHO digit ratios

The correlations between RAD digit ratios and the corresponding WHO ratios, calculated for each side and for males and females separately ($n = 12$ correlation coefficients), were positive (range 0.21–0.69) and statistically significant in all cases ($P < 0.045$), except for the 2D : 3D left ratio of females and the 2D : 4D right ratio of males (both $P > 0.20$). The mean correlation coefficient across all digit ratios was 0.45 (95% CL 0.35–0.55), and the strength of the correlation did not differ among sexes or sides (analysis of variance, effects of sex, $F_{1,9} = 0.09$, $P = 0.77$; side, $F_{1,9} = 0.01$, $P = 0.98$; the non-significant side × sex interaction, $F_{1,8} = 0.37$, $P = 0.56$, was removed from the model).

Sex- and side-related variation in digit ratios

The analysis of RAD digit ratios showed that 2D : 3D and 2D : 4D, but not 3D : 4D, were sexually dimorphic, males showing larger values than females (Table 1). Moreover, the 3D : 4D ratio differed between sides, the right side showing significantly larger values than the left, whereas no side effects for the other ratios could be observed (Table 1).

The patterns of sex- and side differences emerging from the analysis of WHO digit ratios were largely coherent with those derived from the analysis of RAD digit ratios (Table 1). However, analyses of WHO digit ratios revealed

Table 1 Mixed model analysis of sex- and side differences in digit ratios (RAD = digit ratios based on radiographic measurements; WHO = digit ratios based on whole digit measurements). Model derived least-squares (LS) means and effect size (see Statistical analyses) are reported

Digit ratio	Predictor	*F*	df	*P*	LS means (95% CL)	Mean difference (95% CL)	Effect size (95% CL)
2D : 3D$_{RAD}$	Sex	9.68	1, 66	0.003	M: 0.709 (0.704/0.713)	0.010	0.33
					F: 0.699 (0.695/0.704)	(0.003/0.016)	(0.12/0.54)
	Side	3.04	1, 67	0.09	L: 0.705 (0.702/0.709)	0.002	0.08
					R: 0.703 (0.699/0.706)	(0.000/0.005)	(−0.01/0.18)
2D : 4D$_{RAD}$	Sex	6.36	1, 66	0.014	M: 0.964 (0.955/0.973)	0.016	0.28
					F: 0.948 (0.939/0.957)	(0.003/0.029)	(0.06/0.50)
	Side	0.07	1, 67	0.80	L: 0.956 (0.949/0.962)	−0.001	−0.01
					R: 0.956 (0.949/0.963)	(−0.005/0.004)	(−0.09/0.07)
3D : 4D$_{RAD}$	Sex	0.30	1, 66	0.59	M: 1.360 (1.349/1.372)	0.004	0.06
					F: 1.356 (1.344/1.367)	(−0.012/0.021)	(−0.17/0.30)
	Side	4.75	1, 67	0.033	L: 1.355 (1.347/1.364)	−0.005	−0.08
					R: 1.361 (1.352/1.369)	(−0.010/0.000)	(−0.15/−0.01)
2D : 3D$_{WHO}$	Sex	4.13	1, 63	0.046	M: 0.729 (0.723/0.735)	0.008	0.20
					F: 0.721 (0.715/0.727)	(0.000/0.017)	(0.00/0.40)
	Side	0.02	1, 64	0.88	L: 0.725 (0.720/0.730)	0.000	−0.01
					R: 0.725 (0.720/0.730)	(−0.006/0.006)	(−0.16/0.13)
2D : 4D$_{WHO}$	Sex	7.21	1, 63	0.009	M: 0.975 (0.966/0.983)	0.016	0.26
					F: 0.959 (0.951/0.967)	(0.004/0.027)	(0.07/0.45)
	Side	4.03	1, 64	0.049	L: 0.963 (0.956/0.970)	−0.009	−0.15
					R: 0.971 (0.964/0.979)	(−0.017/−0.000)	(−0.29/0.00)
3D : 4D$_{WHO}$	Sex	0.66	1, 63	0.42	M: 1.337 (1.326/1.348)	0.006	0.09
					F: 1.331 (1.320/1.341)	(−0.009/0.022)	(−0.13/0.30)
	Side	5.88	1, 64	0.018	L: 1.328 (1.320/1.337)	−0.011	−0.15
					R: 1.340 (1.331/1.348)	(−0.020/−0.002)	(−0.28/−0.03)

a marginally significant (*P* = 0.049) directional asymmetry in the 2D : 4D ratio, which could not be detected in the corresponding RAD digit ratio (*P* = 0.80) (Table 1). On the other hand, a statistically significant directional asymmetry in 3D : 4D ratios was shown by both analyses, the right side showing larger values than the left (Table 1).

Discussion

Our results provide the first osteometric evidence that 2D : 3D and 2D : 4D ratios are sexually dimorphic in any avian species, males having larger ratios than females. In addition, our study provides the first evidence that digit ratios based on whole digit measurements reliably reflect underlying osteometric digit ratios. Thus, estimates of digit ratios based on whole digits largely convey the same biological information as those based on radiographs. Moreover, right feet were found to have larger ratios than left feet for the 3D : 4D ratio (see also Navarro et al. 2007).

The correlation between RAD and WHO digit ratio estimates (*r* = 0.45, 95% CL 0.36–0.53) compares favourably with the same relationship assessed for the 2D : 4D ratio of a large sample of humans (*r* = 0.45, *n* = 136) (Manning, 2002). Moreover, the extent of sexual dimorphism and side differences in digit ratios evaluated on whole digit measurements was remarkably coherent with radiographic

estimates, as could be shown by comparison of effect sizes (Table 1). However, whole digit measurements highlighted a slightly larger right vs. left 2D : 4D ratio, a pattern which was not detected in radiographic estimates (Table 1). This may suggest minor directional asymmetries in the size or shape of leg scales, which may translate into directional asymmetries in digit ratios independently of actual skeletal asymmetries.

We emphasize that the correlation between RAD and WHO digit ratios could vary in different species according to variation in measurement error, which could be greater for whole digit measurements than for skeletal ones due to variation in soft tissues. Thus, previous claims of a lack of sex dimorphism in digit ratios based on whole digit measurements should be interpreted with caution, as no osteometric validation has been performed (e.g. Forstmeier, 2005). Moreover, the relative importance of measurement error is expected to increase with decreasing body size, thus reducing the power of the statistical tests of sexual dimorphism in digit ratios in small compared to large species.

The finding that avian digit ratios are sexually dimorphic (Burley & Foster, 2004; Saino et al. 2007) has important consequences for the advancement of digit ratio studies. In fact, avian eggs contain maternally derived hormones, whose amount can be experimentally manipulated

(Groothuis et al. 2005). Such manipulations are not feasible in mammals, where the intimate connections between the maternal body and the developing fetus hamper direct manipulations of the *in utero* hormonal environment. As hormones have important activational and organizational effects on morphological, physio-logical, psychological and performance traits, a large body of work has suggested that digit ratios could serve as phenotypic markers of prenatal exposure to androgens and estrogens, by predicting variation in such traits (review in Manning, 2002). Moreover, two recent experimental studies of the pheasant (*Phasianus colchicus*) have shown that prenatal exposure to physio-logical doses of androgens or estrogens affect digit ratios, thus mechanistically linking digit ratio variation to variation in maternal hormonal environment (Romano et al. 2005; Saino et al. 2007), and Navarro et al. (2007) found that the expression of a testosterone-dependent secondary sexual trait (badge size) positively covaried with 2D : 4D ratio in male house sparrows (*Passer domesticus*). These findings suggest that hormone-mediated maternal effects could affect interindividual variation in avian digit ratios, which could thus serve as markers of prenatal steroid exposure (McIntyre, 2006), although the issue remains controversial (Forstmeier, 2005; Garamszegi et al. 2007). Finally, it is interesting to note that the few studies of birds reporting significant sex differences in 2D : 4D ratio (i.e. Burley & Foster, 2004; Navarro et al. 2007; Saino et al. 2007; this study) highlighted a pattern of sexual dimorphism that is opposite to the one observed in mammals. In fact, in mammal species studied to date (with perhaps the single exception of Guinea baboons, *Papio papio*; Roney et al. 2004), males show smaller 2D : 4D ratio than females, whereas in birds the reverse is true. It might be speculated that these differences are related to the different mechanisms of sex determina-tion in birds and mammals because, contrary to mammals, in birds the female is the heterogametic sex. Opposite patterns of sex dimorphism in 2D : 4D ratios in birds and mammals could therefore stem from the control or modulation of digit ratios by sex-linked genes, as originally suggested by Phelps (1952) to explain sex differences in human digit ratios.

In conclusion, the results of this radiographic study show for the first time that sex differences in avian digit ratios have an osteometric basis and are not due to sex-specific variation in soft tissues, thus corroborating the idea that sex differences in digit ratios are related to variation in *Hox* genes expression controlling the development of limb skeletal parts in avian species.

Acknowledgements

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